Defining Structural Requirements for Neuropeptide Y Receptors Using Truncated and Conformationally Restricted Analogues†

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To further elucidate the minimum bioactive conformation of neuropeptide Y (NPY), a series of truncated and conformationally constrained analogues has been prepared. The synthesis and purification of these peptides was achieved using routine laboratory strategies and techniques. Parent molecules consisted of the native NPY N-terminal 1–4 and C-terminal 25–36 segments, having the residue 5–24 core replaced by either a single flexible ω-aminoalkanoic acid, or a more rigid Pro-Gly or Pro-DAla sequence which was expected to constrain a putative turn, and allow the N- and C-termini to align. Cross-linking between residues 2 and 27 through lactamization using side-chain length and chirality suggested by computer simulations, resulted in cyclo-(2/27)-des-AA$^{7-24}$[Glu^{2},Gly^{6},DDpr^{27}]NPY that exhibited very high affinity (K_i = 0.3 versus 0.3 nM for NPY) for the Y_2 receptor using SK-N-BE2 human neuroblastoma cells, yet very low affinity for the Y_1 receptor using SK-N-MC human neuroblastoma cells (K_i = 130 versus 2.0 nM for NPY). The added constraint resulting from bridging in this analogue as well as in others suggested that the combination of the deletion of residues 5–24 and the introduction of an internal ring produced exclusive selectivity for the Y_2 receptor with little or no loss of affinity. The tolerance of structural recognition was further demonstrated as a second ring was introduced which was expected to constrain the amphiphilic α-helix, resulting in the full Y_2 agonist dicyclo (2/27,28/32)-des-AA$^{7-24}$ [Glu^{2,32},DAla^{6},DDpr^{27},Lys^{28}]NPY. Improvement of Y_1 binding activity was achieved only by including more residues (des-AA$^{10-17}$) in the central PP-fold region, while allowing limited flexibility of the termini. Although the length of the bridge seemed to have little effect on binding potency, changes in the location of and chirality at the bridgehead resulted in analogues with different binding affinities. Combination of optimum structural modifications resulted in cyclo-(7/21)-des-AA$^{10-18}$[Cys^{7,21}]NPY, an analogue shortened by 25% but retaining comparable binding properties to that of native NPY at Y_1 and Y_2 receptor types (K_i = 5.1 and 1.3 nM, respectively).

Introduction

Neuropeptide Y (NPY), a 36 residue C-terminally amidated polypeptide,[1,2] elicits numerous physiological effects and has been implicated in the etiology of various disease states, including hypertension, obesity, and various psychiatric disorders.[3-7] NPY is a member of the pancreatic polypeptide (PP) family. The tertiary structure of avian PP (aPP) has been elucidated in detail (<1 Å) by X-ray diffraction analysis revealing an intramolecularly stabilized helical structure referred to as the PP-fold.[8,9] Recent spectral studies as well as molecular dynamics simulation (MDS) suggest that NPY may have a similar intramolecularly stabilized three-dimensional structure; specifically, a polyproline-type II helix for residues 1–8, a β-turn through positions 9–14, an amphiphatic α-helical segment extending from residues 15–32 and a C-terminal turn structure from residues 33–36.[10,11] Recently reported NMR studies of the NPY dimer in solution, however, suggest slightly modified secondary structural characteristics.[12,13]

Neuropeptide Y receptors occur in multiple forms, of which two, designated Y_1 and Y_2 receptors, are the best characterized.[14-17] Receptors of the Y_1 subtype have recently been cloned[18] and exist in the sympathetic nervous system predominantly postjunctionally to mediate vasoconstriction, while those of the Y_2 subtype are postulated to be present prejunctionally to regulate catecholamine release. The distinction between the two types is derived from differential binding properties of C-terminal fragments of NPY, especially the 13–36 fragment which binds only to cells containing Y_2 receptors (SK-N-BE2) whereas [Leu^{31},Pro^{34}]NPY binds almost exclusively to the Y_1 receptor subtype.[15,19]

Since NPY is known to play a significant role in normal physiological and pathological processes, the importance of developing synthetic NPY agonists and antagonists is evident. Before endeavoring to design potent analogues with desired pharmacological properties, it is beneficial to determine the bioactive conformation of the native peptide upon interaction with its receptors. Previous structure–activity relationship (SAR) studies have demonstrated that the amidated C-terminal segment was

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† Abbreviations: The abbreviations for the amino acids are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise. In addition: Aca, 6-aminocaproic (hexanoic) acid; Aoc, 8-aminooctanoic acid; aPP, avian pancreatic polypeptide; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DCHA, dicyclohexylamine; DCM, dichloromethane; DMF, dimethylformamide; Dpr, 2,3-diaminopropionic acid; EDT, ethanedithiol; DIC, 1,3-diisopropylcarbodiimide; Fmoc, (fluoren-9-ylmethoxy)carbonyl HOBt, 1-hydroxybenzotriazole; MBHA, methylbenzhydrylamine; MDS, molecular dynamics simulation; NMP, *N*-methylpyrrolidone; NPY, neuropeptide Y; OFm, fluoren-9-ylmethyl ester PP, pancreatic polypeptide; PYY, peptide YY; SAR, structure–activity relationships; SPPS, solid-phase peptides synthesis; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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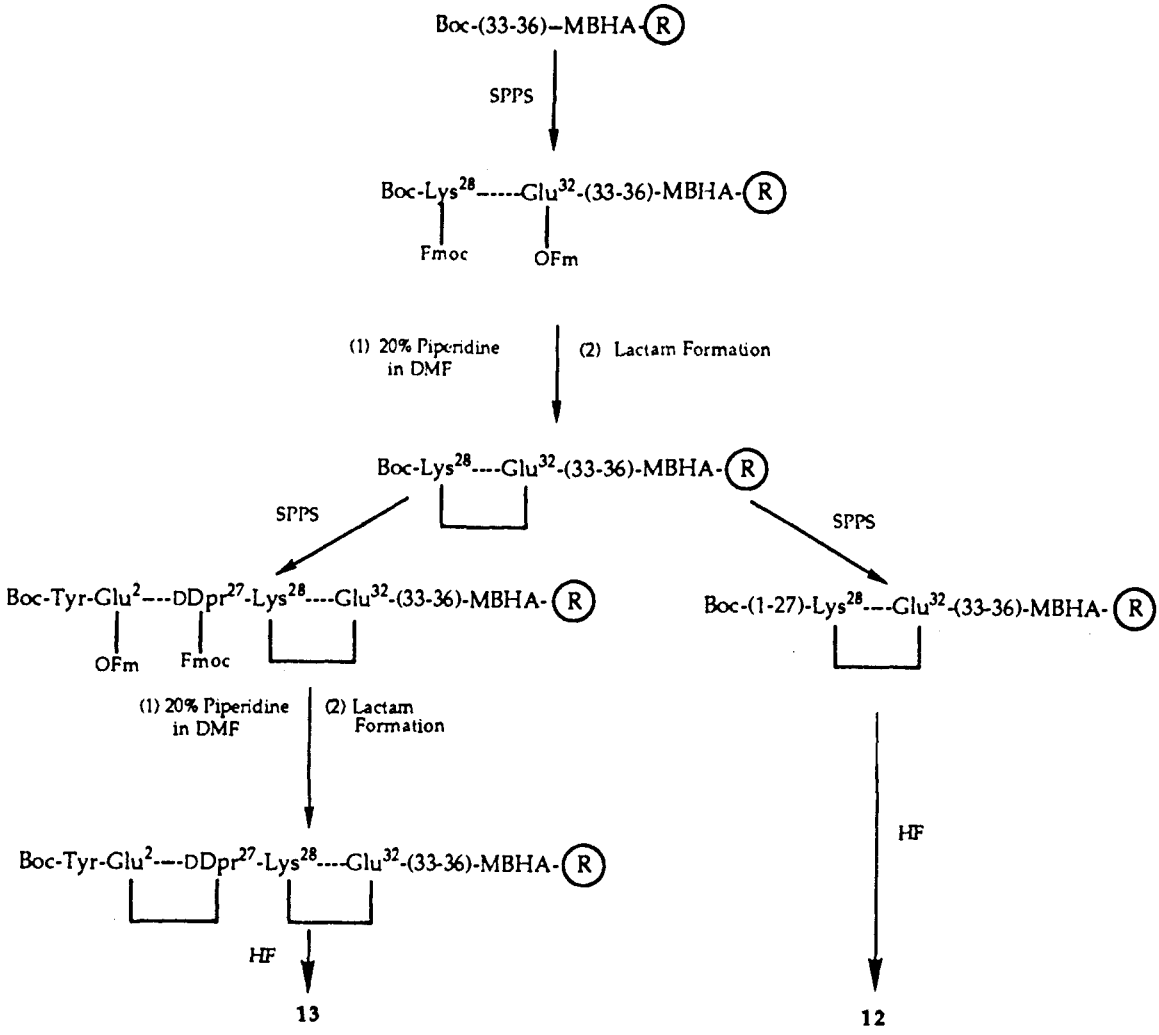


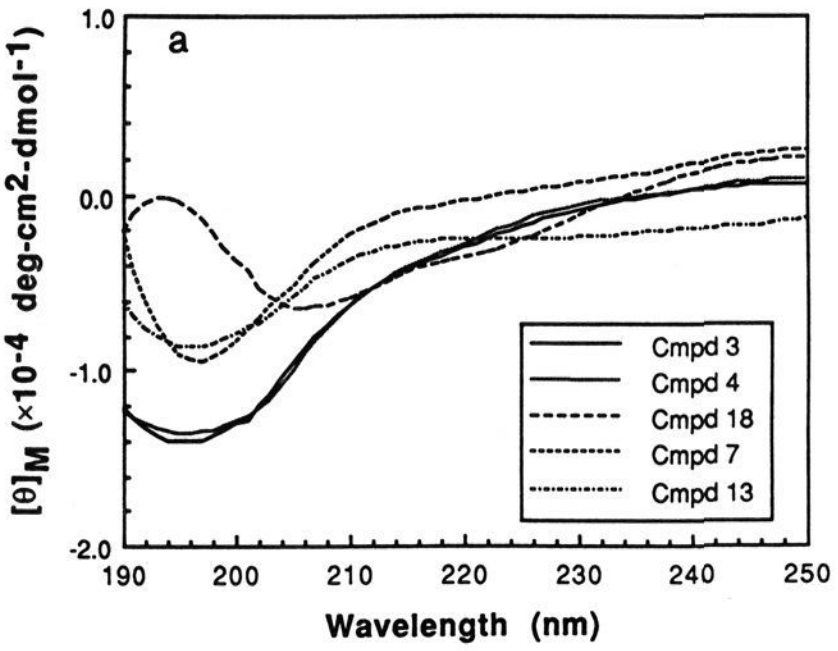
Figure 1. Schematic diagram illustrating synthetic stages leading to cyclic and dicyclic lactam analogues.

essential for receptor binding and biological activity.[19,20] Likewise, it has been shown that residues in the N-terminal portion were also essential for effective interaction of NPY with the Y_1 receptor, with hydrophobic interactions contributing to the bioactivity by stabilizing the proximity of the N- and C-termini.[21,22] It has been postulated that the residues composing the central core of NPY contained only features of structural significance (i.e. helicity, hydrophobicity, intramolecular stability)[21] and may not be essential for direct receptor interaction. Krstenansky et al.[23,24] and Beck-Sickinger et al.[25,26] have produced several centrally truncated analogues in which a flexible spacer was substituted for centrally deleted residues, while retaining substantial bioactivity. Beck-Sickinger further demonstrated the tolerance of the Y_2 type receptor in recognizing analogues which were sterically altered or contained chirally inverted residues. We expand here on previous work,[27] detailing the synthesis and binding activity of a series of centrally truncated analogues in which a potential β-turn partial sequence (Pro-Gly or Pro-DAla) replaces the deleted residues. Design of analogues was derived in part from modeling experiments based on homology to the crystal structure of avian PP together with the results from previous SAR studies. Molecules were further stabilized covalently through the introduction of lactam and disulfide bridges using positions 2, 5, 7, 20, 21, 24 and 27, which have been shown to tolerate substitutions without significant loss of affinity.[23,24,28] Optimization of binding affinities was explored by the manipulation of bridge position, length, and chirality, producing both high affinity Y_2 selective analogues as well as some displaying high affinity at the Y_1 receptor.

Results

Chemical Synthesis. All peptides were assembled using standard solid-phase peptide synthesis (SPPS) techniques. Synthesis of cyclic analogues with disulfide and lactam bridges presents unique challenges. Orthogonal systems of SPPS have been developed by our group and others,[29,30] in which two or more side-chain protection methods are employed. Cyclization via the unprotected side-chain groups proceeded both while the peptide remained attached to the polystyrene resin support and after final cleavage from the resin. Lactam formation was performed on the resin (see Figure 1). Activating reagents such as DIC/HOBt and/or BOP were most successfully employed in conjunction with polar solvents such as DMF or NMP. The repeated use of HOBt, which is suspected to catalyze the deprotection of the imidizole of His(Tos), and the prolonged presence of DIC, however, produced the undesirable formation of a carbodiimide amidine with the imidazole group of His^{26} in significant quantity (>20%), as reported elsewhere.[31] It was possible to cleave this adduct by treating the resin bound and fully protected peptide with hot dilute methanol; no further side reactions (methyl esterification or *O*-methylisourea formation) were detected by mass spectrometric analysis of the major components of the crude preparation.

The reactivity of free sulfhydryl groups of cysteine-containing analogues provided a convenient method for



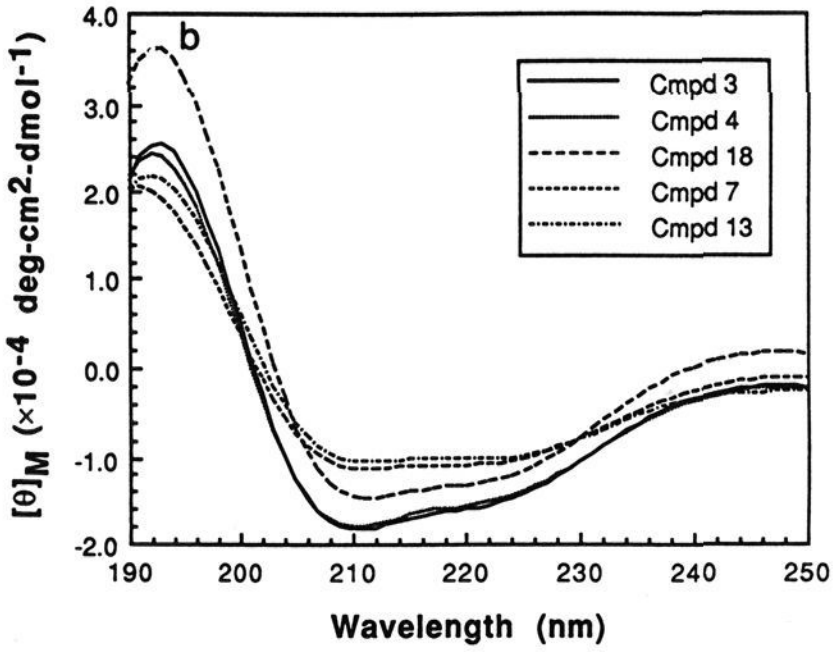


Figure 2. (a) CD spectra of des-AA$^{6-24}$[Ahx^5]NPY (**3**), solid line; des-AA$^{7-24}$[Gly^6]NPY (**4**), dotted line; cyclo(2–27)-des-AA$^{7-24}$[Glu^2,Gly^6,DDpr^{27}]NPY (**7**), small dashed line; dicyclo-(2/27, 28/32)-des-AA$^{7-24}$[Glu^{2,32},DAla^6,DDpr^{27},Lys^{28}]-NPY (**13**), dash-dot line; and cyclo(7–21)-des-AA$^{10-17}$[DCys^7,-Cys^{21}]NPY (**18**), dashed line; all at 100 μM in 0.01 M sodium phosphate, 0.05 M sodium chloride, pH 7.40 buffer. (b) CD spectra of peptides **3**, **4**, **7**, **13**, and **18** in 30% (v/v) TFE. Mixture was formed by dilution of 0.7 mL of peptide solution (described above) with 0.3 mL TFE. Line type is as described for part a.

cyclization and was easily performed by air oxidation. However, because of the oxidative sensitivity and high nucleophilicity of sulfur, it must be noted that cysteine-containing peptides are occasionally known to undergo side reactions, including dimerization and thiol-mediated decomposition reactions.[32] Under high dilution conditions, these undesirable reactions were greatly reduced, and all seven disulfide peptides were obtained in good yield and purity after HPLC purification. Because of the possibility of incomplete cyclization not detected by other means, it was important to have confirmation of composition by mass spectroscopy.

Circular Dichroism Spectra (Figure 2, Table II). The residue molar elipticities in the wavelength range 190–250 nm of des-AA$^{6-24}$[Ahx^5]NPY (**3**), des-AA$^{7-24}$[Gly^6]NPY (**4**), cyclo(2–27)-des-AA$^{7-24}$[Glu^2,Gly^6,DDpr^{27}]NPY (**7**), dicyclo(2/27,28/32)-des-AA$^{7-24}$[Glu^{2,32},DAla^6,DDpr^{27},-Lys^{28}] (**13**), and cyclo(7–21)-des-AA$^{10-17}$[DCys^7,Cys^{21}]NPY (**18**), in aqueous buffer (0.01 M sodium phosphate, 0.05 M sodium chloride, pH 7.40) shown in Figure 2a suggest a strong random coil or turn component for these peptides in aqueous solution, and the lack of any positive elipticity below 200 nm argues against any helical content. De-

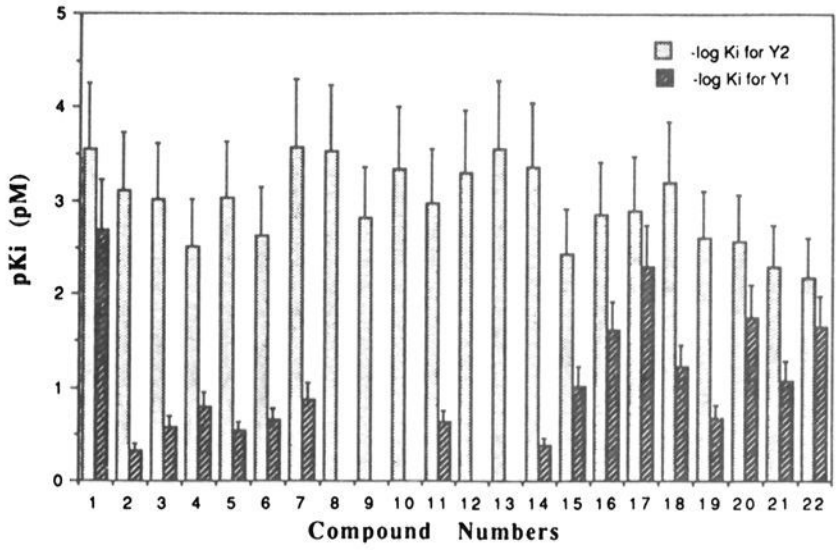


Figure 3. Graphical comparison of receptor binding affinities given as pK_i values in pM, where pK_i = –log K_i. Binding to Y_1 receptors using SK-N-MC human neuroblastoma cells (stripped) and binding to Y_2 receptors using SK-N-BE2 human neuroblastoma cells (grey).

convolution of these spectra (Table II) using the PROSEC program (Aviv Assoc.) shows negligible α helix components for any of the peptides under aqueous conditions. Compounds **3** and **4** have virtually identical spectra under these conditions. The spectra of **3**, **4**, **7**, **13**, and **18** in 30% TFE (Figure 2b) indicate that all five peptides become helical under mixed aqueous–organic conditions. Again, the spectra of compounds **3** and **4** are very similar (helical) despite the elimination of Pro^5 in **3** and replacement of Ahx^5 with Pro^5-Gly^6 in **4**.

Receptor Binding Activity. Y_1 Binding Affinity Using SK-N-MC Human Neuroblastoma Cells (refer to Table I and Figure 3). The human SK-N-MC cell line, which had been reported to express the Y_1 receptor subtype, bound NPY with an apparent K_i of 2.0 ± 0.1 nM. This binding was to a single class of receptors (P = 0.030) numbering approximately 120 000 per cell. The human SK-N-BE2 cell line was similarly investigated. The cells bound NPY to two classes of receptors (P < 0.0001) with apparent K_i's of 0.3 ± 0.1 and 180 ± 110 nM. The high affinity receptors, which had been reported to be of the Y_2 subtype, numbered approximately 25 000 per cell. In contrast, the low affinity class of binding sites were determined to be due to trace being displaced from the plastic microwells. In the absence of cells, the plastic wells displayed what appeared to be saturable nonspecific binding sites with a K_i of 200 ± 100 nM for NPY. This nonspecific displacement was not apparent for the Y_1 receptors because of the greater number (~120 000) of high affinity receptors on the SK-N-MC cells.

Saturation binding experiments were performed for both the SK-N-MC and SK-N-BE2 cell lines using [^{125}I]PYY as the radioligand. The Scatchard plot of the [^{125}I]PYY saturation binding data obtained for the SK-N-BE2 cells was nearly linear. The slope of the line fitting the data was -3.16×10^{10}, corresponding to an affinity constant K_D of 0.032 nM. This was in close agreement with the K_D of 0.080 nM obtained for PYY in competitive binding experiments. The slope of the corresponding Hill plot for the data was equal to 1.03, indicating that no cooperativity existed between the high and low affinity binding sites observed for the SK-N-BE2 cells.

From reports in the literature it was known that the Y_1 and Y_2 NPY receptor subtypes could be differentiated by their abilities to bind C-terminal fragments such as NPY_{13-36}.[34,42] NPY_{13-36} (**2**) displaced [^{125}I]PYY from SK-N-MC cells with a K_i of 460 ± 62 nM. This low affinity

Table I. Sequence, Receptor K_i Values, % Purity, HPLC Retention Times, and Monoisotopic Masses of NPY Analogues

compd no.	compd name {ref} and sequence[a]	Y_1 binding[b] K_i, nM (±SEM)	Y_2 binding[c] K_i, nM (±SEM)	purity[d]	t_R[e]	calcd[f] mass	obsd[g] m/z
1	NPY {1,2}						
	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY	2.0 (±0.1)	0.3 (±0.1)	>99	30.4	4254.74*	4255.0**
2	NPY(13-36) {13}						
	PAEDLARYYSALRHYINLITRQRY	460 (±62)	0.8 (±0.2)	>99	31.9	2981.59	2981.6
3	des-AA$^{6-24}$[Aca^5] {25}						
	YPSKB----------RHYINLITRQRY	261 (±66)	1.0 (±0.2)	>97	11.0	2220.25	2220.5
4	des-AA$^{7-24}$[Gly^6]						
	YPSKPG----------RHYINLITRQRY	160 (±60)	3.1 (±0.5)	>98	11.0	2261.24	2261.1
5	des-AA$^{7-24}$[DAla^6]						
	YPSKPa----------RHYINLITRQRY	290 (±48)	0.9 (±0.2)	>98	11.7	2275.26	2275.5
6	cyclo(2/27)-des-AA$^{7-24}$[Glu^2,Aca^5,DDpr^{27}]						
	YESKB----------RHxINLITRQRY	220 (±36)	2.3 (±0.4)	>98	4.0	2157.22	2157.1
7	cyclo(2/27)-des-AA$^{7-24}$[Glu^2,Gly^6,DDpr^{27}]						
	YESKPG----------RHxINLITRQRY	130 (±26)	0.3 (±0.1)	>98	4.2	2198.21	2198.1
8	cyclo(2/27)-des-AA$^{7-24}$[Glu^2,DAla^6,DDpr^{27}]						
	YESKPa----------RHxINLITRQRY	>1000 (±0.1)	0.3 (±0.1)	>97	6.2	2212.22	2212.0
9	cyclo(2/27)-des-AA$^{7-24}$[Asp^2,DAla^6,DDpr^{27}]						
	YDSKPa----------RHxINLITRQRY	>1000 (±0.1)	1.5 (±0.4)	>95	3.9	2198.21	2198.0
10	cyclo(2/27)-des-AA$^{7-24}$[Asp^2,DAla^6,DLys^{27}]						
	YDSKPa----------RHkINLITRQRY	>1000 (±0.1)	0.5 (±0.1)	>95	7.7	2240.25	2240.2
11	cyclo(2/27)-des-AA$^{7-24}$[DGlu^2,DAla^6,DDpr^{27}]						
	YeSKPa----------RHxINLITRQRY	230 (±61)	1.1 (±0.3)	>99	8.5	2212.22	2212.0
12	cyclo(28/32)-des-AA$^{7-24}$[DAla^6,Lys^{28},Glu^{32}]						
	YPSKPa----------RHYKNLIERQRY	>1000 (±0.1)	0.5 (±0.1)	>98	7.7	2300.25	2300.5
13	dicyclo(2/27,28/32)-des-AA$^{7-24}$[Glu^{2,32},DAla^6,DDpr^{27},Lys^{28}]						
	YESKPa----------RHxKNLIERQRY	>1000 (±0.1)	0.3 (±0.1)	>99	3.2	2237.22	2236.6
14	cyclo(5/24)-des-AA$^{10-18}$[Glu^5,DAla^6,DLys^{24}]						
	YPSKEDNPa----------RYYSAkRHYINLITRQRY	410 (±29)	0.4 NA	>95	12.6	3383.73	3383.3
15	cyclo(7/20)-des-AA$^{10-18}$[Glu^7,DAla^9,DLys^{20}]						
	YPSKPDEPa----------RkYSALRHYINLITRQRY	96 (±40)	3.7 (±2.4)	>95	25.3	3316.78	3316.9
16	cyclo(7/20)-des-AA$^{10-17}$[Cys^7,Cys^{20}]						
	YPSKPDCPG----------ARCYSALRHYINLITRQRY	24 (±2.4)	1.4 (±0.6)	>99	25.0	3338.68	3338.7
17	cyclo(7/21)-des-AA$^{10-17}$[Cys^7,Cys^{21}]						
	YPSKPDCPG----------ARYCSALRHYINLITRQRY	5.1 (±0.6)	1.3 (±0.6)	>95	24.6	3340.86*	3340.4**
18	cyclo(7/21)-des-AA$^{10-17}$[DCys^7,Cys^{21}]						
	YPSKPDcPG----------ARYcSALRHYINLITRQRY	60 (±3)	0.6 (±0.2)	>99	23.2	3338.67	3338.4
19	cyclo(7/21)-des-AA$^{10-17}$[DCys^7,DCys^{21}]						
	YPSKPDcPG----------ARYCSALRHYINLITRQRY	210 (±15)	2.5 (±1.4)	>99	25.4	3338.67	3338.7
20	cyclo(7/20)-des-AA$^{9-17}$[DCys^7,Aoc^8,Cys^{20}] {22}						
	YPSKPDcJ----------RCYSALRHYINLITRQRY	18 (±2)	2.7 (±1.6)	>95	26.7	3325.72	3325.8
21	cyclo(7/21)-des-AA$^{9-17}$[DCys^7,Aoc^8,Cys^{21}]						
	YPSKPDcJ----------RYCSALRHYINLITRQRY	87 (±2.6)	5.2 (±2.3)	>99	25.7	3325.70	3325.7
22	cyclo(7/21)-des-AA$^{9-17}$[Cys^7,Aoc^8,Cys^{21}]						
	YPSKPDCJ----------RYCSALRHYINLITRQRY	22 (±2.2)	6.6 (±3.9)	>97	26.0	3327.91*	3327.4**

[a] Small letters indicate D-amino acids; B, 6-aminocaproic acid; J, 8-aminooctanoic acid; X, 2,3-diaminopropionic acid; all analogues are peptide amides. [b] K_i values in nM using SK-N-MC human neuroblastoma cells, $n = 6$. [c] K_i values in nM using SK-N-BE2 human neuroblastoma cells, $n = 6$. [d] Determined by analytical HPLC. [e] Retention times using linear gradient of 30–65% B in 35 min: solvent A, 0.1% TFA; solvent B, 60% MeCN in A; Vydac C_{18} column (5 μm particle size; 46 × 250 mm); detection: 0.1 AUFS at 210 nm. Flow rate 2.0 mL/min. [f] Calculated monoisotopic mass (except where denoted by * indicating average mass) for $[M + H]^+$. [g] Observed m/z for resolved monoisotopic species (except when denoted by ** indicating unresolved species) measured with LSIMS; glycerol and 3-nitrobenzyl alcohol (1:1) matrix; Cs ion source.

Table II. Calculated Secondary Structural Components for Compounds 3, 4, 7, 13, and 18 in Aqueous and 30% TFE Solutions[a]

compd	P_α	P_β	P_T	P_R
		Aqueous		
3	.00	.29	.20	.51
4	.00	.31	.19	.50
18	.00	.75	.00	.25
7	.00	.51	.12	.37
13	.01	.45	.13	.41
		30% TFE		
3	.42	.00	.24	.34
4	.40	.00	.23	.37
18	.36	.55	.00	.08
7	.24	.44	.05	.27
13	.25	.51	.03	.21

[a] Spectra were deconvoluted using the program PROSEC (Aviv Associates) which employs the reference spectrum of Yang et al.[43]

supported the conclusion that the NPY receptors on the SK-N-MC cell line were of the Y_1 subtype. NPY_{13-36} displaced [^{125}I]PYY with a K_i of 0.80 ± 0.20 nM. This high affinity supported the conclusion that the NPY receptors on the SK-N-BE2 cell line were of the Y_2 subtype.

Des-AA$^{6-24}$[Aca^5]NPY (3) was first tested by Beck-Sickinger et al.[26] using rabbit kidney receptors which are predominantly of the Y_2 type. All analogues in which the central (7–24) residues were removed resulted in greatly impaired Y_1 binding affinity to SK-N-MC cells. In our assay, analogue 3 bound poorly to the Y_1 receptor (K_i = 260 nM), with affinity nearly 130 times less than that of NPY. Binding was improved by almost 2-fold in 4 which incorporated a Pro-Gly link (160 nM), although the binding affinity of 5 with the Pro-DAla substitution was not further improved compared to that of the parent analogue 4. The introduction of a lactam bridge by connecting the side-chain moieties of residue 2 (Glu) to position 27 (DDpr) produced compound 7, with increased binding affinity (130 nM). A modification of this cyclic analogue, in which a DAla was substituted in position 6 (8) resulted in complete loss of affinity at the Y_1 receptor at the highest concentrations tested (10^{-6} M), while changing the chirality of the bridgehead at position 2 resulted in 11 with a K_i = 230 nM. Shortening or elongating the side-chain bridge length

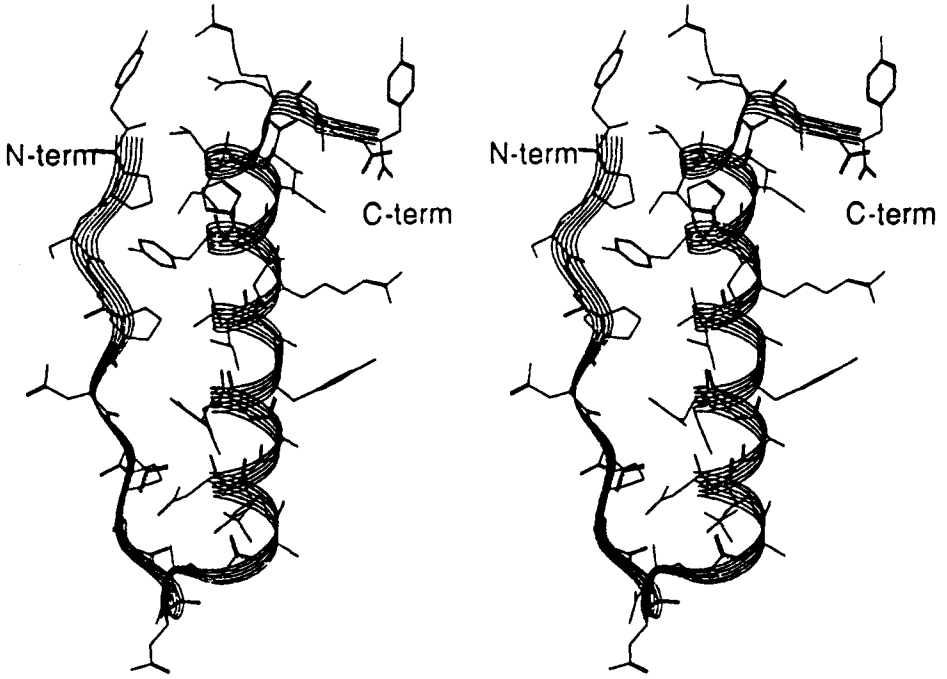


Figure 4. Stereo ribbon illustration of NPY in PP-fold conformation obtained by modeling of NPY by homology to X-ray crystallographic structure of aPP and tethered minimization to relieve side-chain stretch.

in compounds **9** and **10** failed to produce Y_1 binding. Constraining the α-helix rather than the two termini such as in **12**, or introducing two cycles as in **13** again produced two analogues with no affinity for the Y_1 receptor.

Whereas **3–13** were truncated by 18 residues, other analogues (**14–22**) were truncated by 9 residues. One of the latter, **14**, cyclo(5/24)des-AA$^{10-18}$[Glu^5,DAla^6,-DLys^{24}]NPY, bound with poor affinity when compared to **7**, the most potent des-AA$^{7-24}$ cyclic compound. Of similar size, but different bridging arrangement than **14**, compound **15**, (with a position 7 to 20 bridge) had improved Y_1 affinity (96 nM). In contrast to the lactam-containing analogues, cyclo(7/20)-des-AA$^{10-17}$[Cys^{7,20}]NPY (**16**), displayed high affinity for the Y_1 receptor (24 nM). Further increase in binding affinity was achieved (5.1 nM) by changing the bridge to extend from residue 7 to residue 21 in **17**. Inverting the chirality at a single bridgehead (DCys^7) resulted in **18** with lower affinity to the Y_1 receptor (60 nM). When both DCys^7 and DCys^{21} were incorporated (compound **19**), a decrease in affinity (210 nM) was observed.

We then compared the binding affinity of Krstenansky's[23] cyclo(7/20)-des-AA$^{9-17}$[DCys^7,Aoc^8,Cys^{20}]NPY, **20**, to that of similar analogues in which the bridge was modified {cyclo(7/21)-des-AA$^{9-17}$[DCys^7,Aoc^8,Cys^{21}]NPY (**21**), and cyclo(7/21)-des-AA$^{9-17}$[Cys^7,Aoc^8,Cys^{21}]NPY (**22**)} and found them to be similar (18, 87, and 22 nM, respectively).

Y_2 Binding Affinity Using SK-N-BE2 Human Neuroblastoma Cells (refer to Table I and Figure 3). The linear analogues **3–5**, des-AA$^{6-24}$[Aca^5]NPY, des-AA$^{7-24}$[Gly^6]NPY, and des-AA$^{7-24}$[DAla^6]NPY, had affinities in the low nanomolar range (1.0, 3.1, and 0.9 nM). Bridging the termini of the more constrained compounds **7** and **8** produced binding affinities equivalent to that of native NPY (0.3 nM), while the more flexible analogue **6** was 5 times less effective. Shortening the bridge, such as in **9**, decreased Y_2 affinity by more than 5-fold, while lengthening the bridge (**10**) decreased affinity by less than 2-fold. Inverting the chirality of the bridgehead at position 2, (**11**), decreased affinity by more than 3-fold. Constraining the α-helix rather than the termini, (**12**), yielded low nanomolar binding affinity (0.5 nM); a modification utilizing both constraints simultaneously (**13**) produced binding affinity equal to that of NPY. Adding more central residues (des-AA$^{10-18}$) and bridging from position 5 to 24 decreased binding (**14**). Changing the bridge arrangement in compound **15** to cyclo(7/20) gave a 13-fold loss of affinity. Cross-linking via a disulfide bridge in **16–19** gave reduced binding affinities ranging from 0.6 to 2.5 nM. Using 8-aminooctanoic acid to replace centrally deleted residues while manipulating bridgehead chirality produced the least potent analogues **20–22** at the Y_2 receptor (binding affinities: 2.7, 5.17, and 6.6 nM, respectively).

Discussion

Neuropeptide Y is a member of the PP family. Avian PP has been crystallized and its structure determined by X-ray diffraction studies to define what was later referred to as the PP-fold.[8,9] The globular PP-fold is characterized by a β-turn connecting a left-handed polyproline II-type helix and an amphiphilic α-helix. For illustration purposes only, we have modeled NPY by homology to the X-ray structure of PP (Figure 4). The exact role of the PP-fold in NPY has been investigated recently by several groups,[21,33] and it was concluded that the PP-fold was of structural as well as functional importance for the recognition of NPY. Assuming that the role of the PP-fold is to facilitate the presentation of the actual binding pharmacophore (C- and N-termini, in correct spatial alignment),[22,34] we then hypothesized that it might be possible to mimic the folded region by smaller chemical segments. A truncated analogue des-AA$^{6-24}$[Aca^5]NPY, developed by Beck-Sickinger et al.,[25,26] suggested that replacing central residues with a freely rotating hydrocarbon spacer gave a ligand which bound to the rabbit kidney membrane receptor. Increased flexibility in the backbone, however, may contribute to destabilization of the hairpin-like tertiary structure which is maintained by hydrophobic interactions and thought to be essential for bioactivity.[10] When the same analogue was tested in our assays (compound **3**), it was determined that the corre-

sponding Y_1 specific binding affinity was low (K_i = 26 nM) while of relatively high affinity (1.0 nM) at Y_2 receptors using SK-N-MC and SK-N-BE2 human neuroblastoma cells, respectively. We expected that a more constrained structure would be favored by the introduction of Pro-DAla or Pro-Gly sequence in the truncated portion, thus inducing a putative β-turn and possibly mimicking the naturally occurring 3-D structure of NPY. Results for the Pro-Gly containing homologue 4 show improved affinity for the Y_1 receptor type, albeit low affinity (K_i = 160 nM), over that found in the comparable deletion analogue 3 using ϵ-aminocaproic acid as a spacer (see Table I). At the same time, this modification decreased Y_2 binding affinity by 11-fold, suggesting differences in specificity for the Y_1 and Y_2 receptors. Previously, it had been suggested that the Y_2 receptor demanded less structural information than the Y_1 receptor for full binding activity[14,21] and is thus capable of recognizing long C-terminal fragments of NPY. Recently, a high-affinity Y_1 specific ligand was produced by combining the C-terminal hexapeptide of pancreatic polypeptide with the PP-fold of NPY, [Leu^{31},Pro^{34}]NPY.[19] These findings together with our present results, suggest that individual NPY receptor subtypes may provide differing active site environments that are capable of promoting conformational modifications, ultimately leading to preferential recognition. To further test this hypothesis, it was necessary to prepare chemically constrained molecules.

Structural information derived from modeling experiments allowed us to construct several molecules thought to conserve the central β-turn and essential structural features for NPY binding. Since the juxtaposition of the N-terminus to the C-terminus was thought to play a crucial role in receptor recognition, we used α-carbon distances derived from molecular models to explore opportunities for bridging through the side chains of the two termini. Though computer modeling was a useful tool, it was noted that the structures extracted from modeling would not necessarily represent solution conformations. In fact, Saudek and others[12,13] recently reported that the NMR solution structure of the NPY dimer differed significantly from our model derived by homology to the crystal structure of aPP. These authors specifically cited an absence of regular structure (polyproline II helix) in the N-terminal region. However at concentrations tested in our assays, aggregation was not expected. It was shown by modeling that the introduction of the Glu^2-DDpr^{27} amide bridge changed the distance between these residues by only 0.5 Å, from 6.18 Å in the NPY model to 6.75 Å in the cyclo(2/27) analogue. The molecule cyclo(2/27)-des-AA$^{7-24}$[Glu^2,Gly^6,DDpr^{27}]NPY, compound 7, was shown to bind to Y_2 specific cells with affinity equal to that of NPY (K_i = 0.3 nM). Additionally, Y_1 affinity was improved over that of the corresponding linear analogue 4 (130 vs 160 nM). A similar bridge combined with the ϵ-aminocaproic acid spacer 6 retained previous Y_1 affinity, with less affinity for the Y_2 receptor. It is unlikely that the deleterious effect of aminocaproic acid spacer is simply a result of differences in hydrophobicity, since substitution with Pro-DAla (8) produced high Y_2 binding affinity comparable to that of NPY. Beck-Sickinger et al. recently reported a modification of analogue 6 in which the introduction of a bridge extended from residue 2 to 30 produced a Y_2 selective agonist.[28] These results again demonstrate the high sensitivity of each receptor for a specific tertiary conformation.

The introduction of DAla in position 6 of the truncated cyclic analogue 8 resulted in a Y_2 selective analogue devoid of Y_1 receptor affinity, illustrating the sensitivity of the Y_1 receptor for specific stereochemistry. Changing the length of the side-chain bridge (either shorter or longer than 6.75 Å) and repositioning the amide function resulted in less potent Y_2 analogues (9 and 10). Both receptors were most sensitive to changes in chirality of the bridge; using a DGlu^2 to DDpr^{27} bridge arrangement, 11, resulted in decreased Y_2 binding affinity and reestablished minimal affinity at the Y_1 receptor (233 nM).

A second opportunity for conformational restriction was explored by bridging two neighboring residues of the α-helical segment of NPY utilizing a Lys^{28} to Glu^{32} lactam ring. Modeling experiments indicated that the α-carbon distance from residue 28 to 32 would contract from 6.15 to 5.50 Å, despite the introduction of eight intervening atoms in the side chains separating the α carbons. This again resulted in Y_2 affinity nearly equal to that of native NPY, with complete loss of Y_1 affinity. These results reaffirm that essential constraints needed for Y_1 receptor recognition must be contained in the C-terminus and stabilized by both the central α-helix and the N-terminus. An analogue was therefore constructed that simultaneously encompassed both bridges, dicyclo(2/27,28/32)-des-AA$^{7-24}$[Glu^{2,32},DAla^6,DDpr^{27},Lys^{28}]NPY (Figure 5). Binding of this constrained analogue was selective for the Y_2 receptor with identical high affinity (0.3 nM). The high structural tolerance of the Y_2 receptor was demonstrated by this constrained molecule, fully recognizing not only a highly truncated NPY substrate, but also one in which gross structural modifications had been introduced.

Having succeeded in designing truncated and constrained analogues that were capable of expressing full Y_2 binding activity, we turned to the question of characterizing Y_1 receptor binding requirements and thereby restoring Y_1 binding. It was first suspected that including more of the central residues might improve binding, if in fact, the PP-fold or specific central residues served a functional role in receptor interaction and activation. Although Reymond et al.[37] found that the linear des-AA$^{10-17}$-[Ala^9]NPY showed relatively little improvement in Y_1 binding (100 nM) compared to the des-AA$^{7-24}$ compounds this may indicate that either more residues would be required or the conformation would need to be adjusted to accommodate a central truncation. In pursuing the latter possibility, the incorporation of a single lactam bridge (cyclo 5/24) was expected to provide stability with minimal disruption of the tertiary structure, but analogue 14 produced 4 times weaker Y_1 binding affinity than the linear des-AA$^{10-17}$ compound. By transposing the bridge more centrally, to form cyclo 7/20, 15 suggested that more flexibility in the combined terminal region improved Y_1 affinity (96 nM). Changing the chemical nature of the bridge to encompass a disulfide linkage and using only L-amino acids produced 16 with enhanced binding affinity (24 nM). Similar analogues incorporating 8-aminooctanoic acid as a spacer were developed by Krstenansky et al. and shown to be potent NPY agonists;[23] the compound cyclo(7/20)-des-AA$^{9-17}$[DCys^7,Aoc^8,Cys^{20}]NPY in our assays gave a Y_1 receptor K_i = 18 nM. It had been shown that modifications of tyrosine at position 20, either by substitution or chiral inversion, led to reduced Y_1 binding affinity.[35] Specifically, the oxygen moiety of the benzyl ring had been shown to be important for adequate receptor binding. In the same study, it was shown that the tyrosine

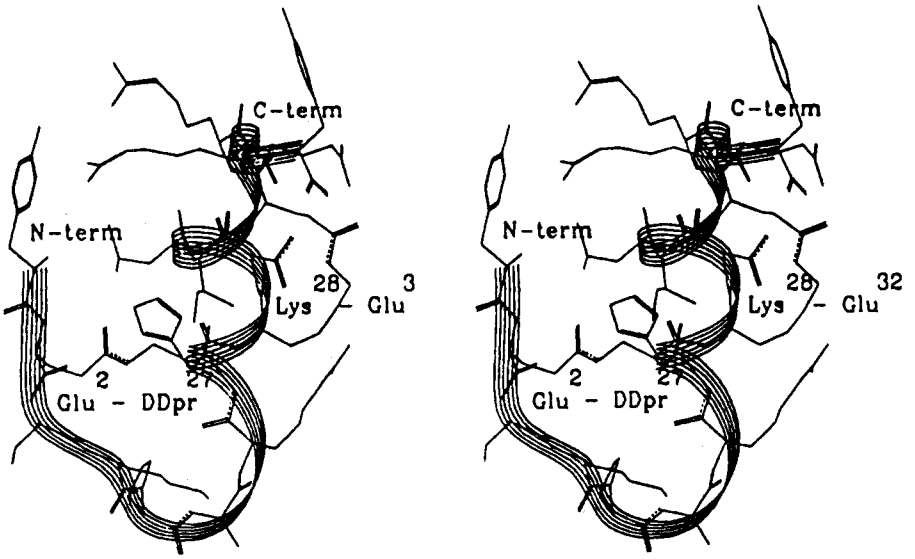


Figure 5. Stereo ribbon illustration of dicyclo(2/27, 28/32)-des-AA$^{7-24}$[Glu^{2,32},DAla^{6},DDpr^{27},Lys^{28}]NPY (**13**) showing amide bridge at residues Glu^{2}-DDpr^{27} and Lys^{28}-Glu^{32}. This conformation was modeled by homology to the working model of NPY (Figure 4).

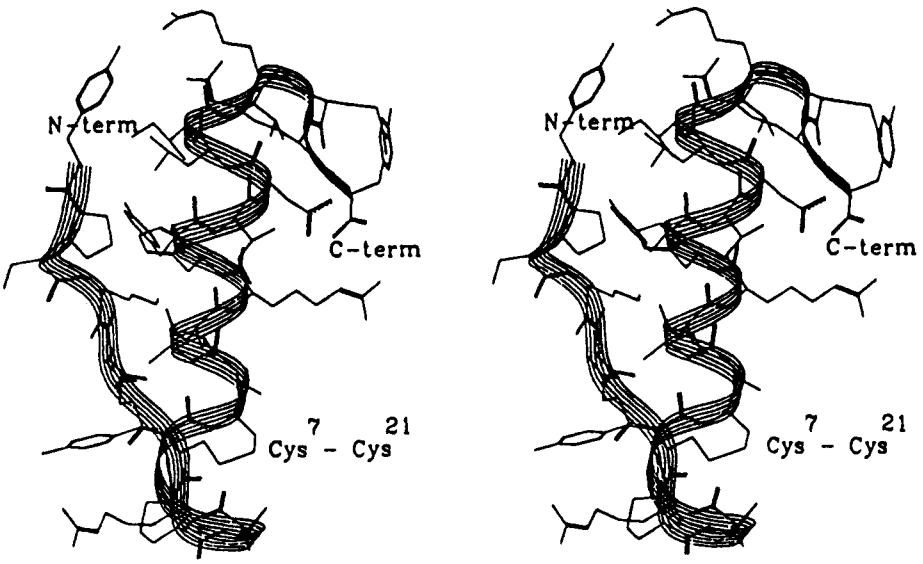


Figure 6. Stereo ribbon illustration of cyclo (7/21)-des-AA$^{10-17}$[Cys^{7,21}]NPY (**17**) built by homology to the working model of NPY (Figure 4) with minimization to relieve strain caused by introduction of the Cys^{7}-Cys^{21} disulfide bridge.

in position 21 played a lesser role in proper receptor interaction. With this in mind, we explored the possibility of extending the disulfide bridge from residue 7 to 21 rather than residue 20, despite computational information suggestion that greater distortion of the molecule would result. Using this bridging arrangement, we observed lower affinity for **21** than we did for Krstenansky-type analogue **20**. However adjusting the chirality of the bridgehead to reduce distortion restored the binding activity of **22** to the level of **20**. By replacing the flexible aminooctanoic acid by the rigid Pro-Gly spacer previously proven beneficial in the lactam series, a short cyclic NPY analogue **17** (model shown in Figure 6) was produced that was nearly equipotent with NPY at both Y_1 and Y_2 receptors (5.1 and 1.3 nM, respectively). In addition, the CD spectrum of des-AA$^{10-17}$[DCys^{7},Cys^{21}]NPY (**18**) under both aqueous, and especially conditions that imitate the hydrophobic nature of the membrane interface (30% TFE), showed definite α-helical character (Figure 2a,b, Table II). Together, these results indicate that the helical core of NPY functions only to stabilize the pharmacophore at the receptor surface and, since specific residues were not required, could successfully be mimicked by shortened, constrained modifications.

We conclude that constrained, deletion analogues in which major central truncation is combined with the bridge connecting residues 2 to 27 are highly preferential for the Y_2 receptor subtype and display affinity equivalent to that of NPY. Since it is known that the Y_2 receptor is the predominant subtype found in the central nervous system, and has been implicated in the attenuation of noradrenaline release as well as in the mediation of the inhibition of adenylate cyclase,[16,36] Y_2 specific agonists will certainly be of great value for future studies. Although binding affinity at the Y_1 receptor is reduced by central truncation, affinity is entirely lost as the placement of the conformational restraint approaches the extreme termini (cyclo 2/27 or 2/30), an observation also noted by Beck-Sickinger et al.[26] and Reymond et al.,[37] whereas a more centrally located bridge (cyclo 7/20 or 7/21) is well-tolerated. These results suggest that enhancement of Y_1 receptor recognition is not dependent on residues composing the entire central α-helical segment, since high Y_1 affinity can be established while removing 25% of the naturally occurring residues and imposing conformational constraints. The Y_2 receptor is most tolerant of major changes in tertiary conformation, but remains sensitive to different parameters than those affecting the Y_1 receptor, since high

affinity Y_1 agonists were in general the poorest Y_2 agonists. Together, these results indicate that the binding requirements of the Y_1 receptor differs greatly from those of the Y_2 receptor, although sharing many features in common.

Experimental Section

All reagents and solvents were of analytical grade (Aldrich Chemical Co., Milwaukee, WI; Fisher Scientific, Springfield, NJ) and were used without further purification except TFA (Halocarbon, Hackensack, NJ) and TEA (Aldrich), which were reagent grade and used without further purification for syntheses and were distilled to constant boiling point for use in chromatographic buffers.

Peptide Synthesis. All peptides were synthesized manually using the Boc-strategy on MBHA resins prepared in our laboratory by methods previously described.[20] Side-chain protection of α-Boc amino acids (Bachem, Torrance, CA) was as follows: Arg(Tos), Asp(β-OcHx or β-OFm), Cys(S-p-Mob), Dpr-(Fmoc), Glu(γ-OcHx or γ-OFm), His(Tos)·DCHA, Lys(2ClZ or Fmoc), Ser(Bzl), Thr(Bzl), and Tyr(2BrZ). Asn and Gln were coupled in the presence of a 2-fold excess of 1-hydroxybenzotriazole. His(Tos)·DCHA was coupled with a 2-fold excess of BOP in the presence of diisopropylethylamine. Removal of the Boc group was accomplished by treatment of the peptide-resin with 60% TFA in DCM in the presence of 1% EDT. The protected peptide-resins were cleaved in anhydrous HF in the presence of 3% anisole at 0 °C for 1.5 h. Crude peptides were precipitated and washed with diethyl ether, then extracted from the resin and other organic material with water, and lyophilized.

Cyclization. Compounds containing lactam bridges were assembled using Fmoc side-chain protection of the β-amino group of DDpr and the fluorenyl methyl ester protection of the γ-carboxyl of Glu, which were removed by two treatments of 20% piperidine in DMF for 10 and 15 min. Lactam formation then proceeded on the resin by the method of Felix et al.[30] in the presence of 3 equiv of BOP and diisopropylethylamine or BOP/HOBt/diisopropylethylamine in DMF for 12–48 h. Completeness of coupling was monitored every 8 h with ninhydrin after thorough washings using DCM, MeOH, and DCM. If incomplete, fresh coupling reagents were added. For more difficult bridge formations, DIC/HOBt in DCM or DMF were utilized as a secondary coupling reagent.

Cyclization of free sulfhydryl-containing analogues was performed following HF cleavage by stirring a dilute solution of the peptide (250 mg/L) in 0.07M NH_4OAc (pH 6.8) at 4 °C for 24 h. Completion of cyclization was monitored by the Ellman test and HPLC. Upon complete oxidation, peptide solution was concentrated on a Bio-Rex-70 column (100 mL) and was eluted with 40% HOAc. A powder was obtained after lyophilization of the diluted peptide-containing fractions.

Purification. Crude peptides were purified by preparative reverse-phase HPLC[38,39] on a Waters DeltaPrep LC 3000 system equipped with a Waters 1000 Prep Pak Module and a Shimadzu SPD-6A variable wavelength UV detector. The cartridges used were hand packed in-house in Waters polyethylene sleeves and frits and Vydac C_{18} packing material (15–20 μm particle size, 30 nm pore size). The material was eluted (95 mL/min) using a linear TEAP (buffer A)/60% MeCN in TEAP (pH 2.25 or 5.2) (buffer B) gradient; acceptable fractions were pooled, reloaded onto the preparative cartridge, and desalted in 0.1% TFA/MeCN. Final products were >95% pure by HPLC analysis.

Peptide Characterization. Purified peptides were subjected to HPLC analysis [Vydac C_{18} column, on a Perkin-Elmer Series 400 Liquid Chromatograph, Kratos Spectroflow 757 UV detector, and Hewlett-Packard Model 3390A integrator, (for specific conditions, see Table I)], amino acid analysis [hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h, followed by ion-exchange chromatography and postcolumn derivatization with o-phthaldehyde], and LSIMS analysis measured with a JEOL JMS-HX110 double focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. Samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. Daughter ion spectra of carboimide amidine analogues were measured using a linked field scan at constant B/E ratio.

Dicyclo(2/27,28/32)-des-AA$^{7-24}$[Glu^{2,32},DAla^6,DDpr^{27},-Lys^{28}]NPY. MBHA resin (4 g, 0.38 mmol NH_2/g) was used to assemble the peptide. Complete couplings as monitored by Kaiser's ninhydrin test were obtained within 2 h for most residues. Ile^{31}, Lys(Fmoc)^{28}, DDpr(Fmoc)^{27}, and Lys(2ClZ)^4 required a recoupling step. Boc and Fmoc groups were removed as described earlier. Formation of the first bridge (cyclo 28/32) was accomplished in 48 h and four treatments with fresh coupling reagents at room temperature (BOP/HOBt/diisopropylethylamine, DIC/HOBt, BOP/diisopropylethylamine and DIC) while the second bridge (cyclo 2/27) required a single treatment with BOP/diisopropylethylamine for 8 h before yielding a negative ninhydrin test. A total of 7.1 g of fully protected peptide-resin was obtained and cleaved by HF as described earlier to give 2.3 g of crude product. Purification was performed in three stages using TEAP, pH 2.25 (gradient from 10 to 30% B in 60 min, retention time ca. 16 min), TEAP, pH 5.2 (gradient from 10 to 30% B in 60 min, retention time ca. 12 min), and 0.1% TFA (gradient from 0 to 30% B in 90 min, retention time ca. 56 min) in the presence of MeCN with a flow rate of 95 mL/min. Yield was 213 mg (6.2% of expected amount from original substitution of the MBHA resin) of final peptide with of purity >98% by analytical HPLC. Synthesis of other cyclic analogues proceeded in a similar manner.

Molecular Modeling. Preliminary working models of the compounds described here were constructed by homology to the crystallographic structure of aPP obtained from the Brookhaven Protein Data Bank. Hydrogen atoms were explicitly added using standard bond distances and valence angles. Following interactive computer graphic analysis and manual manipulation to eliminate severe overlap (Insight II, BIOSYM Technologies), the structures were minimized with a restraining potential employed to tether the backbone atoms using DISCOVER (BIOSYM) with the CVFF force field of Hagler et al.[40,41] Finally, the tethering potential was relaxed and the NPY model was allowed to undergo flexible geometry minimization. The models illustrated here (Figures 4–6) do not necessarily represent the solution conformations of these analogues and are included for their heuristic value.

Circular Dichroism Spectra. Circular dichroism (CD) measurements were obtained with an Aviv Model 62DS spectropolarimeter (Aviv Associates, Lakewood, NJ) under control of the manufacturer's operating system (60DS) using 0.5-mm cuvettes thermostated at 20 °C and signal averaging four scans in the range 190–250 nm. Data were collected at 1.0-nm intervals with a 2.0-s integration time and a spectral bandwidth of 2.0 nm. Spectra of compounds **3**, **4**, **7**, **13**, and **18** were collected under two sets of buffer conditions: (1) in 0.01 M sodium phosphate, 0.05 M sodium chloride (pH 7.40), and (2) the above buffer diluted 70/30 (v/v) with 2,2,2-trifluoroethanol. Concentrations were based on the calculated molecular weight of the TFA salt of the purified lyophilized peptide assuming a water content of 7% and were used for the calculation of residue molar elipticities; water content was not routinely determined. Residue molar elipticities were calculated based on the number of residues in each analogue, irrespective of the presence of side chain–side chain amide bonds.

Cell Culture. Receptor binding assays were performed using the human neuroblastoma SK-N-MC cells (Y_1 receptors) and the SK-N-BE(2) cells (Y_2 receptors). Cells were grown to confluence in 100-mL plates, trypsinized, and then plated into 24 microwells plates (10^5 cells per well). Media for SK-N-MC cells was MEM Eagles (500 mL) buffer, containing L-glutamine (10 mL, 200 mM), penicillin/streptomycin (5 mL, 10 000 mg/mL), nonessential amino acids (5 mL, 100X Irvine Scientific), Na + pyruvate (5 mL, 11 mg/mL), fetal calf serum (50 mL, 10%), and 500 μL of fungiyorl. Media for SK-N-BE(2) cells was DME-F12 (500 mL) buffer, containing L-glutamine (10 mL, 200 mM), penicillin/streptomycin (5 mL, 10 000 mg/mL), fetal calf serum (50 mL, 10%), and 500 μL of fungiyorl. After 1 day for the SK-N-BE(2) and 2 days for the SK-N-MC, the cells had grown to the appropriate density and were used for binding assays.

Binding Experiments. Peptides to be tested were preliquoted at 100 μg/mL in 100 μL of solution in Milli-Q water (Millipore, U.S.A.). Solutions of peptides in different concentrations (seven doses from 10^{-6} to 10^{-10} M) were prepared in binding buffer (0.30 M sucrose, 10 mM HEPES, 0.1% BSA, and pH is adjusted to 7.4 with NaOH). Microwell plates were precoated with p(Lys_2-Ala) (100 μL of 0.2 g/L) in doubly distilled H_2O at 37 °C for 1 min followed by replacement with 400 μL of binding buffer per well and stored at 37 °C until all plates had

been pretreated. Trace ([^{125}I]PYY from NEN, 2200 Ci/mmol) was diluted to 15 000 cpm/50 μL. Each peptide solution (50 μL) was tested in triplicate in the presence of 50 μL of trace. Nonspecific binding was determined in the presence of 10^{-6} M cold peptide. After 45 min of incubation at 37 °C, the cells were washed with 250 μL of ice-cold binding buffer and lysed with lysis buffer (8 M urea, 3 M acetic acid, 2% triton). For each well, buffer was transferred to a tube for counting. The content of each well was counted using a Micromedic γ counter. Binding data was analyzed using the nonlinear regression analysis program LIGAND [Biosoft, McPhearson modification (1985) of original method of Munson & Robard] which iteratively optimized variables (included standard error) describing a sigmoid curve.

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